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Genomic Regions of Coxsackievirus B3 Associated With Cardiovirulence

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The molecular basis for cardiovirulence in the coxsackievirus B3 (CVB3) genome was examined in a murine model of acute myocarditis. Infectious cDNAs representing a highly cardiovirulent coxsackievirus B3 ($CVB3_m$) and a noncardiovirulent ($CVB3_0$) virus were used to construct infectious chimeric cDNAs. Assays of the resulting recombinant viruses for cardiovirulence in adolescent male CD-1 mice showed that the 5′ nontranslated region (5′ NTR) of the $CVB3_m$ genome plays the major role in determining cardiovirulence and that the genomic region encoding the capsid proteins has a minor additive effect in increasing cardiovirulence. Nucleotide sequences in the 5′ NTR of $CVB3_m$ and $CVB3_0$ differ at 23 positions; 14 are located in four stem-loop motifs of the secondary structure and may influence the cardiovirulent phenotype by regulating RNA or protein synthesis. A comparison of predicted amino acid sequences of capsid proteins in $CVB3_m$ and $CVB3_0$ identified two amino acids as potential candidate contributors to cardiovirulence, i.e., amino acids at positions A207 (Asn-Asp) in the puff structure of the E-F loop of VP2 and A566 (Gln-Glu) in the C terminal of VP3 at the external surface. The data from this study and published literature support the conclusion that cardiovirulence of a CVB3 can depend on several regions of the genome. ***J. Med. Virol. 52:341–347, 1997.***

KEY WORDS: coxsackieviruses group B; recombinant viruses; chimeric viruses; mice; myocarditis

INTRODUCTION: STUDYING HUMAN PATHOGENS

Coxsackieviruses are well-established human pathogens that replicate in many different organs, such as heart, pancreas, kidney, lung, brain, gastrointestinal mucosa, and liver. Infections by the virus may be severe and even lethal, although most cases are subclinical or mild [Grist and Reid, 1988]. Coxsackievirus B3 (CVB3) murine models of myocarditis have provided considerable insight into mechanisms of induction of an inflammatory disease of the heart and the role of host genetics, age, or gender on the outcomes of infection [Gauntt et al., 1993; Leslie et al., 1989; Rose et al., 1992]. In addition, stress on the CVB3-infected host can affect the outcome [Beck et al., 1994; Lerner et al., 1975]. To better understand the pathogenesis of the disease, several mouse model systems and strains of CVB3 have been studied [Gauntt et al., 1979, 1993]. Two CVB3 strains have received extensive study relative to the genetic basis of cardiovirulence: $CVB3_m$ is highly cardiovirulent in mice and $CVB3_0$ is noncardiovirulent [Gauntt et al., 1979]. These variants could not be distinguished from each other on the basis of titers of infectious virus produced in murine heart tissues in vivo [Gauntt et al., 1979]. The molecular basis for cardiovirulence/attenuation in the $CVB3_0$ strain has been determined: a U at position N234 of the 5′ nontranslated region (NTR) dictates cardiovirulence, whereas a C renders the virus noncardiovirulent [Tu et al., 1995]. Conservation of the immediate adjacent sequence (GG*C*UA) around N234 in the 5′ NTR of most enterovirus genomes may indicate an essential function of this region that when altered results in loss of virulence [Tracy et al., 1996]. A number of other nucleotide differences exist between genomic sequences of $CVB3_0$ and another cardiovirulent CVB3 [Chapman et al., 1994; Tracy et al., 1991, 1992], suggesting that additional nucleotide sites or sequences may be important cardiovirulence determinants in other CVB3 strains. A correlation was found between the presence of an A at position N565 and cardioviru-

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lence in three CVB3 strains versus a C or U at this position in 12 noncardiovirulent CVB3 strains [Gauntt and Pallansch, 1996].

To study the genetic basis of virulence in picornaviruses, recombinant chimeric viruses have been constructed from cDNA clones of virulent and avirulent viruses and tested in animal model challenge experiments [Almond, 1987; Minor, 1992; Tracy et al., 1991; Wimmer et al., 1993]. Chimeric viruses generated from genomic sequences of an avirulent CVB4 strain and a pancreovirulent CVB4 strain were used to map the genetic determinants for murine pancreovirulence to a single change at amino acid A129 within the predicted exterior D-E loop of capsid protein VP1 [Caggana et al., 1993] and to a lesser extent, a replacement of a serine with an arginine at A16 in VP4 [Ramsingh and Collins, 1995]. In polioviruses, a primary attenuating genetic locus has been mapped using vaccine strain genomes: a single transition in the region of N472-481 in the 5′ NTR accounts for a significant amount of attenuation in all three serotypes of poliovirus Sabin vaccine strains [Almond, 1987; Evans et al., 1985; Macadam et al., 1991; Racaniello, 1988; Wimmer et al., 1993]. Other major sites of attenuation map to amino acid changes in the capsid protein-encoding genes [Almond, 1987; Racaniello, 1988] or one of the latter genes and the 5′ NTR [Ren et al., 1991; Westrop et al., 1989; Wimmer et al., 1993]. The virulence phenotype of the more distantly related cardioviruses is also affected by alterations in the 5′ NTR nucleotide sequence and in the sequence encoding capsid proteins [Palmenberg, 1989]. The aim of this study was to generate recombinant viruses from restriction fragments of infectious cDNAs of $CVB3_0$ and $CVB3_m$ to identify a region(s) associated with cardiovirulence in mice.

MATERIALS AND METHODS

Recombinant Viruses Generation

Construction of recombinant viruses proceeded as follows. $CVB3_m$ RNA was obtained from purified virus particles by a standard extraction method [Chapman et al., 1994]. cDNA was synthesized using the Superscript cDNA synthesis system (BRL). Subclones of $CVB3_m$ and $CVB3_0$ were inserted into the phagemid pBluescript II KS (Stratagene) for the purpose of recombinant cDNA construction (Fig. 1). $CVB3_0$ cDNA was constructed by combining PCR amplified fragments using specific restriction endonuclease sites [Chapman et al., 1994]. Recombinant cDNAs were generated by ligating the 5′ NTR fragment (NI through N537) to other sequence fragments from either $CVB3_m$ or $CVB3_0$ (Fig. 1). Another approach used restriction endonucleases that have one or two unique recognition sites, either in the multiple cloning site of the plasmid or in the viral cDNAs. Four subclones were used for the construction of the full length recombinant cDNA clones (Fig. 1). One pair of clones contain the 5′ NTR fragments of $CVB3_m$ or $CVB3_0$ cDNA and the 3D and 3′ NTR of $CVB3_m$ cDNA as PstI-NotI fragments. The subclone containing a portion of the 5′ NTR of $CVB3_0$ as a NotI-PstI fragment is designated pCO354 and the subclone containing a sequence from the 5′ NTR of $CVB3_m$ as MluI-PstI fragments is designated pCM348 (Fig. 1). The other pair of clones contain the entire $CVB3_m$ or $CVB3_0$ cDNA, except the region encoding the carboxy terminal portion of VP1, 2A, 2B, and the N-terminal portion of 2C. The subclone carrying the whole $CVB3_m$ genome except the latter sequence, which represents an internal EcoRI fragment, was designated pCM351, and the corresponding subclone containing $CVB3_0$ sequences except the latter sequence was designated pCO362. The internal PstI fragment of the $CVB3_m$ cDNA was inserted into the subclone pCO354 to construct vCB357. vCB308 was constructed by ligating pCM348, minus the 3D and 3′ NTR sequence, with a 6.9kb PstI-ClaI fragment of the $CVB3_0$ genome. vCB360 consists of pCM351 and the internal EcoRI fragment from the $CVB3_0$ cDNA, and vCB365 was constructed by inserting the corresponding EcoRI $CVB3_m$ cDNA fragment into pCO362. vCB370 was constructed by ligating the 3.3kb EcoRI-ClaI fragment of $CVB3_0$ with a 5.7kb fragment of pCM351 and the internal EcoRI fragment of $CVB3_m$ cDNA. vCB303 was generated by ligation of the 6.8kb PstI-EcoRI fragment of pCO362 with the 2.2kb PstI-EcoRI fragment of the vCB370 clone and then the internal EcoRI fragment of $CVB3_0$ cDNA was inserted. vCB304 was generated by

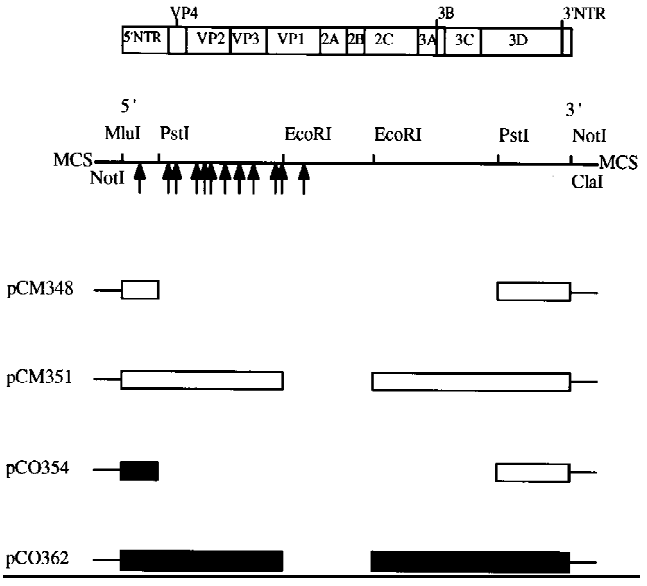

Fig. 1. Schematic representation of cDNA subclones and restriction endonuclease recognition sites used in constructing chimeric CVB3 clones. The top box depicts the structural organization of the CVB3 viral genome. NotI and ClaI at the bottom of the partial restriction map were used to swap the 5′ NTR and 3′ terminus fragments of $CVB3_0$ subclones. The PstI site at the 3′ end of the partial restriction map exists only in the $CVB3_m$ genome. Open and filled boxes correspond to sequences originating from $CVB3_m$ or $CVB3_0$ cDNA, respectively. Short single lines extending from the partial restriction map and the boxes represent multiple cloning sites of plasmid vector pBluescript II, which is not shown. Arrows indicate positions where mutations were identified in the 5′ NTR (Tu et al., 1995) and the capsid protein genes that differentiate $CVB3_m$ from $CVB3_0$: N234 (Tracy et al., 1996), N830, N893, N987, N1271, N1361, N1682, N1874, N2438, N2585, N2726, and N2990.

substituting the $CVB3_m$ PstI-ClaI fragment from vCB370 for the same $CVB3_0$ sequence. vCB307 utilized the 1 kb ApaI-BsaI fragment containing all of VP1 and the sequence encoding ~200 amino acids from the amino terminus of 2A from $CVB3_m$ inserted into the cDNA of $CVB3_0$.

Nucleotide Sequence Analysis

The nucleotide sequence of $CVB3_m$ cDNA was determined by the dideoxy chain termination method [Sanger et al., 1977]. To prepare single-stranded DNA templates, the recombinant plasmid containing the $CVB3_m$ cDNA was denatured with 0.2 M NaOH and 0.2 mM EDTA for 30 min at 37°C. This DNA was precipitated with ethanol, dried and sequenced according to the procedure of U.S. Biochemical Corporation (Arlington Height, IL), using CVB3-specific primers [Chapman et al., 1990, 1994] and sequenase 2 (U.S. Biochemical Corp.).

Cells and Propagation of Recombinant Viruses

HeLa cells were used for virus propagation and plaque assays, as previously described [Gauntt et al., 1979]. To produce infectious recombinant CVB3, positive sense viral genomic RNA was transcribed from each cDNA using T7 RNA polymerase. The transcripts were mixed with DEAE-dextran to 200 μg/ml and 0.2 ml added to 80% confluent HeLa cell monolayers per well in 12 well plates, and incubated for 4 hr at 37°C in a humidified 5% CO_2 incubator. The transfection mixtures were removed and virus growth medium (1% fetal calf serum, streptomycin, penicillin and MEM) was added. Incubation was continued for 48 hr. Each recombinant CVB3 was plaque-purified three times in HeLa cells and stocks prepared in HeLa cells. Progenitor and recombinant viruses recovered from HeLa cells exhibited indistinguishable plaque morphology and size.

Cardiovirulence Assays of CVB3 Recombinants in Mice

Cardiovirulence of recombinant molecular CVB3 viruses was assessed using virus diluted in Dulbecco's phosphate-buffered saline (DPBS) to 1×10^5 plaque-forming units (pfu) in 0.2 ml and inoculated intraperitoneally into groups of five adolescent male CD-1 mice (4–6 weeks old) on day 0. Deeply anesthesized (Metafane) mice were sacrificed on day 7 postinoculation (p.i.) by cervical dislocation. Two coronal sections of each heart were stained with H&E and the number of focal lesions in the myocardium enumerated at 100× [Gauntt et al., 1993]. A lesion scoring system for this study is as follows (mean number per section): ++++, >50 lesions; +++, 20–49 lesions; +, 1–3 lesions and +/–, 0–1 lesion.

RESULTS

Cardiovirulence Maps to Two Regions of the Genome

Cardiovirulence was mapped to the 5′ NTR and capsid protein-encoding region of the CVB3 genome by the following data. Coronal heart tissue sections, evaluated for number of focal myocardial lesions, confirmed that virus from the parental molecular $CVB3_m$ clone was highly cardiovirulent, whereas virus from the parental molecular $CVB3_0$ clone was relatively noncardiovirulent (Fig. 2). vCB370 was as cardiovirulent as the $CVB3_m$ clone, suggesting that the 3′ terminal portion of the CVB3 genome, beginning with a portion of 2C is not associated with cardiovirulence. vCB360 contains an insert of capsid protein VP1, 2A, 2B, and part of 2C genes from $CVB3_0$ and is also highly cardiovirulent. In reverse, viral vCB365 carries most of the VP1, 2A, 2B, and some 2C sequences of $CVB3_m$ inserted into a $CVB3_0$ background and did not induce significant cardiovirulence. vCB357 contains the 5′ NTR of $CVB3_0$ joined to the remainder of the $CVB3_m$ genome, and this construct also exhibited significantly decreased cardiovirulence. vCB308, constructed with the 5′ NTR of the $CVB3_m$ ligated to the remainder of the $CVB3_0$ genome recovered most, but not all of the cardiovirulence phenotype, suggesting a small contribution by a nucleotide or nucleotide sequence downstream of N537 within the 5′ NTR, or outside of the 5′ NTR, to cardiovirulence.

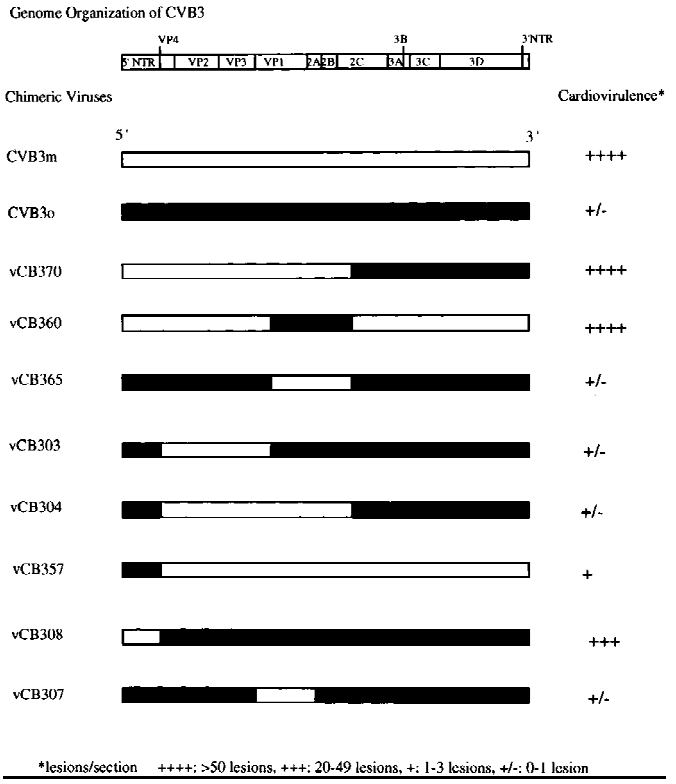

Fig. 2. Genomic construction and cardiovirulence of progenitor and chimeric CVB3 viruses. Recombinant CVB3 clones were constructed using convenient restriction endonuclease cleavage sites located within the 5′ NTR or in VP1 or the nonstructural 2B protein encoding sequences in $CVB3_m$ and $CVB3_0$. After recovery of infectious viral clones from the HeLa cell lysates, the identity of parent and recombinant viruses were verified by neutralization-inhibition assays. Recombinant CVB3 clones were tested for cardiovirulence in male adolescent CD-1 mice. Open boxes correspond to genomic sequences derived from the highly cardiovirulent $CVB3_m$ strain and filled boxes from the noncardiovirulent $CVB3_0$ strain.

TABLE I. Nucleotide and Predicted Amino Acid Sequence Differences in the 5′ NTR and Capsid Polypeptides-encoding Regions Between $CVB3_m$ and $CVB3_0$ Strains

Nucleotide number	$CVB3_m$ nucleotide/ A.A.		$CVB3_0$ nucleotide/ A.A.		Amino acid number
VP4 (N743-949)					
830	A	Ile	G	Val	30
893	A	Ser	G	Gly	51
VP2 (N950-1738)					
987	T	Val	C	Ala	82
1271	A	Ile	G	Val	187
1361	A	Asn	G	Asp	207
1682	A	Ile	G	Val	314
VP3 (N1739-2452)					
1874	G	Val	A	Ile	378
2438	C	Gln	G	Glu	566
VP1 (N2453-3304)					
2585	A	Ser	G	Gly	615
2726	A	Ile	T	Leu	662
2990	G	Val	A	Ile	750

Whole or parts of the capsid protein-encoding sequence of the cardiovirulent $CVB3_m$ genome inserted into the $CVB3_0$ background did not totally recover the cardiovirulence phenotype, as shown by lesion count data with recombinant viruses vCB303 and vCB304. However, inclusion of the 5′ NTR and most of the capsid protein-encoding region of $CVB3_m$ with the remainder of the $CVB3_0$ genome (vCB370 and vCB360) recovered the complete cardiovirulence phenotype exhibited by $CVB3_m$. vCB307 contains the $CVB3_m$ sequence for VP1 plus a few nucleotides into the 2A gene inserted into a genomic background of $CVB3_0$ and was essentially noncardiovirulent.

Candidate Amino Acids Potentially Associated With Cardiovirulence

Predicted amino acid sequences of capsid proteins of $CVB3_m$ were compared to those of $CVB3_0$, to determine possible candidate amino acids associated with cardiovirulence in CVB3 (Table I). Capsid proteins of $CVB3_m$ and $CVB3_0$ have amino acid differences at 11 positions. Two amino acid changes at positions A30 (I-V) and A51 (S-G) occur in VP4, the smallest capsid proteins located entirely in the viral interior [Hogle et al., 1985; Rossman et al., 1985]; however, neither amino acid is near the predicted cleavage site between VP4 and VP2 [Palmenberg et al., 1989]. Four amino acids vary between $CVB3_m$ and $CVB3_0$ in VP2: A82 (V-A), A187 (I-V), A207 (N-D), and A314 (I-V): three involve conservative hydrophobic differences, but one difference at position A207 (N-D) changes the net charge and is exposed on the E-F loop ("puff") of VP2, the largest and most variable loop on the surface of the virus particle [Muckelbauer et al., 1995]. The E-F ("puff") loop of VP2 contains one neutralizing site of CVB3 [Beatrice et al., 1980] and is a site of attenuation in polioviruses [Minor et al., 1993]. Capsid protein VP3 differentiates $CVB3_m$ from $CVB3_0$ at position A566 (Q-E) and is five residues upstream of the putative VP3/VP1 cleavage site [Klump et al., 1990; Tracy et al., 1992] where VP1 joins with the neighboring protomer. The A566 position in the canyon may be involved in receptor recognition or in a neutralizing antibody-binding site [Muckelbauer et al., 1995]. In VP1, a change at position A615 (S-G) alters polarity of the amino acid and is inside the viral capsid. Differences in VP1 are in residues A615, A662, and A750 in the β-sandwich pocket that is involved in binding the antiviral drug WIN66393 [Muckelbauer et al., 1995]. Position A615 is in the interior and A662 is located next to the external B-C loop of the protein by alignment analysis with crystallographic data from the three-dimensional capsid structures derived for $CVB3_m$ [Muckelbauer et al., 1995].

Nucleotides Associated With Cardiovirulence

These results suggest that a nucleotide(s) other than N234 within the initial 537 nucleotides of the 5′NTR of $CVB3_m$ is a necessary major determinant of the cardiovirulent phenotype. A different potential cardiovirulence site in the 5′ NTR was reported at N565, a nucleotide within the internal ribosome entry site (IRES), in a study of naturally occurring isolates from patients that proved to be either cardiovirulent or noncardiovirulent in mice [Gauntt and Pallansch, 1996]. This position does not contribute to cardiovirulence in the $CVB3_m$/$CVB3_0$ recombinants, as both $CVB3_m$ and $CVB3_0$ have an A at N565. If the IRES is involved in cardiovirulence of CVB3, a cis-acting element in the 5′ NTR of $CVB3_m$ may be interacting with N565, and mutations in the cis-acting element would affect protein synthesis and, consequently, cardiovirulence. As shown in Table II, there are 23 nucleotide differences, including two downstream of N537 in predicted stemloop motifs F and G that are associated with the IRES. Because cardiovirulence is not as completely expressed by vCB308, compared to $CVB3_m$, N565 could be one of several nucleotides that is important in cardiovirulence. However, the 5′ NTR fragment of $CVB3_m$, within the background of $CVB3_0$ (vCB308), did not completely recover the cardiovirulence phenotype of $CVB3_m$. The opposite construct (vCB357) showed that ligation of the $CVB3_0$ 5′ NTR fragment to the remainder of the $CVB3_m$ genome considerably reduced the cardiovirulence-inducing capacity, but not to the level of $CVB3_0$. Fourteen of the 23 nucleotide changes between $CVB3_m$ and $CVB3_0$ within the 5′ NTR can be found in secondary structure stemloop motifs predicted by at least one of three computer models (Table II). Three nucleotide differences were detected within the first 90 nucleotides, a region implicated in poliovirus RNA replication, possibly via binding viral and cellular proteins [Haller and Semler, 1995]. Two nucleotide changes at N125 and N157 are located in stemloop C or a small stem (2); this motif interacts with eIF-2 alpha [Haller and Semler, 1995]. Nine nucleotide changes within N234–N395 are found in motif SM1 [Le and Zuker, 1990], E [Haller and Semler, 1995], or motif 5 [Skinner et al., 1989], a region known to form a ribonucleoprotein complex with at least two HeLa cell proteins. Two

TABLE II. Nucleotide Differences Between $CVB3_m$ and $CVB3_0$ in the 5′ NTR Sequence and Their Positions in Computer-Predicted RNA Secondary Structure Stemloop Motifs

Nucleotide number	$CVB3_m$ nucleotide	$CVB3_0$ nucleotide	Location in predicted stemloop motifs		
			Ref. 1[a]	Ref. 2[b]	Ref. 3[c]
31	C	U	1	A	1
38	U	C	—[d]	—	—
90	U	C	—	—	—
125	U	A	2	C	—
157	U	C	2	C	3
234	U	C[e]	—	E	—
261	C	U	SM1	E	5
271	A	G	SM1	E	5
273	A	G	SM1	E	5
276	G	A	SM1	E	5
315	C	U	SM1	E	5
320	C	U	SM1	E	5
380	U	C	SM1	E	5
395	A	G	SM1	E	5
446	A	G	—	E	—
499	A	G	SM2	F	6
598	C	U	SM3	G	—
645	U	A	—	—	—
647	U	C	—	—	—
677	A	G	—	—	—
697	G	A	—	—	—
728	A	G	—	—	—
738	A	G	—	—	—

[a]CVB1 sequence, Le and Zuker [1990] J. Mol. Biol. 216, 729–741.
[b]PV1 sequence, Haller and Semler [1995] Virology 206, 923–934.
[c]PV3 sequence, Skinner et al. [1989] J. Mol. Biol. 207, 379–392.
[d]Nucleotide not located in predicted secondary stemloop motif.
[e]Found in the cardiovirulent $CVB3_0$ strain [Tu et al., 1995].

nucleotide changes at N499 and N598 are located in SM3 [Le and Zuker, 1990] or stemloop motifs F and G, motifs that contain portions of the internal ribosome entry site and also involve the putative binding of one, but not likely two [Haller and Semler, 1995], additional HeLa cell protein [Jackson and Kaminski, 1995]. Thus, these 14 nucleotide changes may be involved directly or indirectly in regulating viral RNA and/or protein synthesis, and influence the cardiovirulence phenotype. Site-directed mutagenesis of nucleotides within the 5′ NTR that differentiate between $CVB3_m$ and $CVB3_0$ genomes will allow defining the significance of each to cardiovirulence.

DISCUSSION

These data suggest that the 5′ NTR and a sequence or nucleotide(s) within the capsid protein-encoding region are required for full expression of cardiovirulence in $CVB3_m$. The primary contribution to the cardiovirulent phenotype derives from the 5′ NTR, specifically within the sequence from the 5′ terminus through N537 as shown by cardiovirulence assays with vCB308, a construct containing most of the 5′ NTR sequence of $CVB3_m$, ligated with the remainder of the $CVB3_0$, genome. The work of Tu et al. [1995] clearly showed that transition of a C to U at N234 resulted in a cardiovirulent phenotype in the $CVB3_0$ strain. Enterovirus sequences logged in GenBank show that most human enteroviruses, including several strains of CVB3 that differ in cardiovirulence phenotypes, contain a U at N234 (Gauntt and Pallansch, 1996; Tracy et al., 1996). Recombinant viruses have been constructed with a $CVB3_0$ genome background and a 5′ NTR derived from noncardiovirulent strains CVB3-DO or CVB3-CO for 4–6-week-old CD-1 mice [Gauntt and Pallansch, 1996; Tracy and Gauntt, 1987]; however, CVB3-DO was found recently to be cardiovirulent in weanling C3H/HeJ male mice [Chapman et al., 1996]. This difference again proves that age and strain are major factors in CVB3-murine models of myocarditis [Gauntt et al., 1993]. As expected, the CVB3-CO/$CVB3_0$ recombinant was noncardiovirulent, but not expected was the finding that the CVB3-DO/$CVB3_0$ recombinant was also noncardiovirulent in weanling C3H/HeJ male mice in which CVB3-DO was highly cardiovirulent. Therefore, replacement of the $CVB3_0$ 5′ NTR with a 5′ NTR from a cardiovirulent CVB3 strain for young C3H/HeJ mice does not always result in gain of a cardiovirulent phenotype.

Studies with these chimeric $CVB3_0$/$CVB3_m$ viruses suggested a conclusion that is in contrast with results derived from two other cardiovirulent CVB3 [Zhang et al., 1993]. An attenuated variant of the CVB3 (Kandolf strain), derived by 14 passages in human dermatofibroblasts, was examined for nucleotide differences from the parent virus in the 5′ NTR. A single point mutation at N690 was found, but when the 5′-terminal 891 nucleotides of the attenuated virus were inserted in place of the wild-type virus sequence in the parent virus genome, the recombinant virus remained cardio-

virulent. These data suggested that the 5′ NTR and most of VP4 do not contain major determinants of attenuation from cardiovirulence [Zhang et al., 1993]. A monoclonal antibody-escape mutant of a cardiovirulent CVB3 strain with significantly decreased cardiovirulence [Van Houten et al., 1991] was recently molecularly cloned, along with the parent virus [Knowlton et al., 1996]. The attenuated variant cDNA differed from the parent virus cDNA in a single nucleotide (N1442) that resulted in an amino acid change (asparagine-to-aspartic acid) at position 165 in the E-F "puff" loop of VP2. Mutations at this position in both parental and mutant virus that led to interchanges of these two amino acids confirmed that asparagine at this position is associated with cardiovirulence [Knowlton et al., 1996]. Thus in two cardiovirulent CVB3 strains, attenuation is not associated with the 5′ NTR.

The differences in amino acids at positions A51 of protein VP4 and A615 of protein VP1 between $CVB3_m$ and $CVB3_0$ may play an important role in sensitization of inflammatory T cells that participate in the pathogenesis of CVB3-induced $CD8^+$ T lymphocytes. It has been reported that cytolytic T lymphocyte (CTL) isolated from spleens of animals inoculated with the cardiovirulent $CVB3_m$ lysed $CVB3_m$-infected target cells better than CTL from $CVB3_0$-inoculated mice [Huber and Job, 1983]. Thus the recombinant CVB3 viruses described above may be useful in assessing contributions of T cells to cardiopathology in CVB3-induced murine models of myocarditis.

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